PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 37/62, 37/26, 37/10

A1

(11) International Publication Number: WO 94/19007

(43) International Publication Date: 1 September 1994 (01.09.94)

(21) International Application Number: PCT/US94/01618

(22) International Filing Date: 14 February 1994 (14.02.94)

(30) Priority Data:
08/017,726 16 February 1993 (16.02.93) US

(71) Applicant: ENZON, INC. [US/US]; 40 Kingsbridge Road, Piscataway, NJ 08854 (US).

(72) Inventors: HALLAHAN, Terrace, W.; 82 Hazelwood Avenue, Metuchen, NJ 08840 (US). GILBERT, Carl, W.; 26 Hampton Court, Basking Ridge, NJ 07920 (US).

(74) Agents: MERCANTI, Michael, N. et al.; Enzon, Inc., 40 Kingsbridge Road, Piscataway, NJ 08854 (US).

(81) Designated States: AU, BG, BR, CA, CZ, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, NZ, PL, PT, RO, RU, SE, SK, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RIBOSOME INACTIVATING PROTEIN COMPOSITIONS HAVING REDUCED ANTIGENICITY

(57) Abstract

Compositions having substances with ribosome-inactivating effect such as trichosanthin are disclosed in combination with non-antigenic polymers, such as poly(ethylene glycol). The compositions are substantially less antigenic than unmodified proteins. Also disclosed are methods of forming the novel compositions of this invention.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

RIBOSOME INACTIVATING PROTEIN COMPOSITIONS HAVING REDUCED ANTIGENICITY

The present invention is directed to substances having ribosome inactivating effects such as that produced by trichosanthin in a substantially non-immunogenic form.

BACKGROUND OF THE INVENTION

Trichosanthin is a plant protein which inhibits protein synthesis via ribosome inactivation. Trichosanthin has an extensive history in oriental medicine and has been shown to have anti-viral activity including activity against the human immunodeficiency virus (HIV). Trichosanthin formulations to date, however, have had serious shortcomings. The protein, often referred to as a ribosome inactivating protein or RIP, is very antigenic, toxic, poorly soluble, and has a short circulating half life in vivo. However, its potential use as a therapeutic agent cannot be ignored. It would be highly desirable to provide ribosome inactivating agents which are more soluble, less antigenic and capable of circulating for longer time periods than trichosanthin.

SUMMARY OF INVENTION

One embodiment of the present invention comprises a modified form of trichosanthin which advantageously has a lower toxicity, increased circulating half life, increased solubility and reduced immunogenicity than heretofore available. This embodiment of the present invention comprises the plant protein trichosanthin which

has been modified with a non-antigenic polymer. One preferred embodiment comprises a conjugate of trichosanthin and a poly(alkylene oxide).

Another preferred embodiment comprises a conjugate of trichosanthin and an average of about 3-5 strands of poly(ethylene glycol) (PEG) per trichosanthin molecule. It has been demonstrated that the conjugates are more soluble than their unmodified counterparts yet retain a sufficient amount of ribosome activation activity and circulate for extended time periods in vivo.

Another embodiment of the present invention comprises a method of modifying a substance having a ribosome inactivating effect such as trichosanthin, in a manner which controls the average number of polymeric strands such as PEG bound to the substance. It has been found that it is desirable to control the degree of protein modification to maximize retention of the ribosome inactivation activity of the conjugate.

According to one embodiment of the present invention, trichosanthin is reacted with a molar excess of a suitable activated polyalkylene oxide such as methoxypoly(ethylene glycol)-N-succinimidyl carbonate (SC-PEG) under conditions sufficient to effect conjugation while maintaining at least a portion of the original trichosanthin activity. Such conditions include reacting the substituents at temperatures of up to about 27°C and in pharmaceutically acceptable buffer systems. As used herein, the term "molar excess" is meant to indicate the ratio of the number of moles of polymeric reagent to the number of moles of trichosanthin. These and other embodiments of the present invention are described in further detail below.

DETAILED DESCRIPTION OF THE INVENTION

One embodiment of the present invention provides a conjugate of substances having a ribosome inhibiting effect such as trichosanthin and a substantially non-antigenic polymer. The non-antigenic polymer advantageously moderates the antigenicity of the protein. Trichosanthin is a plant protein having a molecular weight of about 25,000 with 9 lysine residues. It is a single chain ribosome inactivating protein (RIP) (Type I Ribosome inactivating protein) isolated from the root tubers of Trichosanthin kirilowii maxim. The protein is a specific RNA N-glycosidase, inactivating eukaryotic ribosomes by hydrolyzing the N-C glycosidic bond of adenine 4324 of the 28S rRNA. This cleavage removes the adenine base from the ribose but leaves the phosphodiester bond intact.

Also within the scope of this invention are proteins that catalytically inactivate ribosomes in a manner similar to tricosanthin. The type I ribosome inactivating proteins are widely distributed in plants. They are usually single polypeptide chains with molecular weights near 30,000 and are generally homologous to the A-chain of ricin. Examples include, pokeweed antiviral protein, gelonin, Alpha- and Beta-momorcharin and the sapoins among many others. See, for example, Immunotoxins, Frankel, Ed. 175-209 (1988). The exact type of substance having the ribosome inhibiting effect is not limited to those substances listed herein. Those of ordinary skill in the art will realize that proteins or other substances having the desired ribosome inactivating effect are understood to be included herein.

The substantially non-antigenic polymer substances included in the conjugates are preferably poly(alkylene

oxides). Within this group of substances are alpha-substituted polyalkylene oxide derivatives such as C_1-C_4 alkyl groups. It is preferred, however, that the non-antigenic material be a monomethyl-substituted PEG homopolymer. Alternative polymers such as other polyethylene glycol homopolymers, polypropylene glycol homopolymers, other alkyl-polyethylene oxides, bis-polyethylene oxides and co-polymers or block co-polymers of poly(alkylene oxides) are also useful. In those aspects of the invention where PEG-based polymers are used, it is preferred that they have molecular weights of from about 200 to about 10,000. Molecular weights of about 2,000 to 7,000 are preferred and molecular weights of about 5,000 are preferred.

Covalent modification of the substance is preferred to provide the hydrolysis-resistant conjugate. The covalent modification reaction includes reacting a substance having the desired activity against ribosomal protein production with a substantially non-antigenic polymer substance under conditions efficient to effect conjugation while maintaining at least a portion of the original activity.

The polymers may be activated in order to effect the desired linkage with the protein substance. By activation, it is understood by those of ordinary skill in the art that the polymer is functionalized to include a desired reactive group. Examples of such activation are disclosed in U.S. patents 4,179,337 and 5,122,614, which are hereby incorporated by reference. In the disclosures of these patents, the hydroxyl end groups of polyalkylene glycols are converted and activated into reactive functional groups.

According to one preferred embodiment, trichosanthin is modified with SC-PEG such as that disclosed in the

'614 patent, supra. This particularly preferred activated form of PEG for use in the present invention is poly(ethylene glycol)-N-succinimide carbamate. This activated polymer forms stable, hydrolysis-resistant carbamate (urethane) linkages with amino groups of the protein. Other activated PEG's such as PEG-isocyanate are also of use. While the references incorporated herein describe epsilon amino group modifications of lysine, other conjugation methods are also contemplated. Aspartic, glutamic and/or arginine or other amino acids as well as carbohydrate modifications where available are also within the scope of the present invention. Covalent linkage by any atom between the protein and polymer is possible. Moreover, non-covalent conjugation such as lipophilic or hydrophilic interactions are also contemplated.

One preferred embodiment of the present invention comprises methods of forming conjugates of trichosanthin or similarly acting proteins and non-antigenic polymers, in a controlled manner which limits the average number of polymer strands linked to about 2-5 per protein. In this aspect, the molar ratio of non-antigenic polymer reagent to trichosanthin is modulated to limit the average number of polymers per trichosanthin molecule. A preferred molar ratio is from about 3-7:1. Controlling this ratio is important in order to balance between loss of ribosome inactivating activity and the desired characteristics of a PEG-modification, i.e. increased serum circulating half life and decreased antigenicity and immunogenicity.

According to one method of the present invention, commercially available trichosanthin is preferably first dialyzed extensively against an appropriate salt buffer system. For example, trichosanthin can be dialyzed against solutions comprising 50 mM sodium phosphate and

100 Mm sodium chloride having a pH of about 7.5. Alternatively, the phosphate buffer having a pH of about 7.5 may be replaced with a borate buffer having a pH of about 8.0 - 9.0.

Following dialysis, an excess amount of pyridoxal phosphate, for example a 1-15 fold molar excess, is mixed with the trichosanthin in order to protect the most reactive lysine sites on the protein. Pyridoxal phosphate forms a Shiff-base with reactive lysine residues in a reaction that is freely reversible. Therefore, after conjugation, the pyridoxal phosphate can be easily removed to deprotect the lysines by simply dialyzing the reaction mixture. The pyridoxal phosphate and trichosanthin are allowed to react, for a sufficient time of about 15 minutes, and preferably for about 10 minutes. This reaction is preferably conducted at moderate temperatures, for example below about 27°C, preferably on ice, to avoid risking damage to the protein.

According to this embodiment, a molar excess of a lysine-modifying non-antigenic polymer, such as a poly(alkylene oxide) e.g. a poly(ethylene glycol), is then added to the trichosanthin/pyridoxal phosphate mixture. The polymeric excess will range from about 3 to about 50 fold molar ratio excess and preferably from about 3 to about 7 fold molar excess of the polymer to the protein. The reaction is preferably carried out at temperatures of from 2 - 10°C. The non-antigenic polymer is allowed to incubate in order to react with the other unprotected lysine sites. The incubation time is dependent upon temperature. For example, a lysine-modifying PEG may be allowed to incubate in an ice bath for a half hour followed by further incubation at room temperature for a half hour. Alternatively, this

incubation could be conducted totally on ice for about two hours. The reaction is then stopped such as by adding a sufficient molar excess of a material which is capable of quickly reacting with the lysine-modifying polymer such as glycine.

After dialysis to remove the excess reagents, the reaction products are further separated for example, by either gel permeation chromatography or by cation exchange fractionation. For example, cation exchange fractionation can be performed on a column of CM-Sephadex gel which has been equilibrated with about 10 mM sodium phosphate pH 6.0. Alternative cation exchange resins may also be used. The PEG-modified trichosanthin protein is eluted from the gel utilizing the same buffer and a suitable eluant, for example sodium chloride.

The elution is preferably performed step-wise. For example, 10 mM sodium phosphate buffers containing 0, 50 and 100 millimoles of sodium chloride are used sequentially as the eluants. The conjugates eluted in the fraction coming off without sodium chloride were extensively modified by the polymer and demonstrated low levels of activity. A desirable fraction can be obtained with the eluant containing about 50 millimoles of sodium chloride. Conjugates of trichosanthin with a sufficient amount of ribosomal inactivation activity having, on the average, about 3-5 strands of PEG have been obtained with this fraction. Essentially unmodified proteins were obtained with the eluant containing 100 millimoles of sodium chloride.

By varying the amount of salt in the eluant, the artisan can selectively elute conjugates containing varying amounts of polymer strands attached to the protein. Thus, according to need, it is possible to obtain a spectrum of therapeutic conjugates. They can

range from highly modified, relatively low activity conjugates with rather long circulating lives to slightly modified, relatively high activity conjugates with somewhat shorter circulating lives, yet, nonetheless, much longer than unmodified proteins.

Another aspect of the present invention provides methods of treatment for Acquired Immunodeficiency Disease Syndrome (AIDS) patients with the human immunodeficiency virus (HIV). The method includes administering an effective amount of the compositions described herein. Those of ordinary skill in the art will realize that the amount of the conjugate used in the method of the present invention will vary somewhat from patient to patient, however, conjugates capable of delivering from about 2 micrograms/kg to about 40 micrograms/kg per day of the protein are contemplated. The optimal dosing of the conjugate can be determined from clinical experience. Moreover, the conjugates are administered in pharmaceutically acceptable vehicles, such as parenteral solutions or other dosage forms.

EXAMPLES

The following examples serve to provide further appreciation of the invention but are not meant in any way to restrict the effective scope of the invention.

Examples 1-3

For these examples, a highly purified form of trichosanthin was obtained from Genelabs Technologies Inc., Redwood City, CA. and was first dialyzed (4 x 100 ml) over night against 0.1M sodium borate pH 8.0. Three reactions corresponding to examples 1-3 respectively,

were set up in which 3, 5, and 20 - fold molar excesses of solid SC-PEG, prepared in accordance with the disclosure of U.S. Patent No. 5,122,614, were added. The reaction mixtures were mixed to dissolve the SC-PEG and placed on ice for 30- min., then at room temperature for 30 min. with occasional stirring. The reactions were stopped by the addition of excess glycine. Excess reagents were removed by dialysis against 0.1M sodium phosphate pH 7.5 and the desired conjugates were purified by gel permeation chromatography. An estimate of the number of PEG molecules attached to the protein (PEG#) was made by determining the number of free lysine residues in the conjugate as compared to the unmodified protein using a TNBS assay. The PEG number for the three modification reactions were estimated to be 2.5 for example 1, 4.2 for example 2 and 6.9 for example 3. respectively. The activities of the conjugates vs. an unmodified trichosanthin control in an in vitro protein synthesis inhibition assay are shown in Table I.

TABLE I

IN VITRO PROTEIN SYNTHESIS INHIBITION ASSAY

Sample	IC50 (ug/ml)
Control	0.000045
Example 1	0.0085
Example 2	0.1
Example 3	10.0

As can be seen from the Table, although activity was retained after modification, the in vitro activity of the protein in the modified form was substantially less than the control.

Examples 4-6

In these examples, attempts were made to increase the protein activity using pyridoxal phosphate prior to PEG modification. The protein was dialyzed against 0.1 M sodium borate pH 8.0 overnight (2 x 1000 ml) overnight. Three reactions corresponding to Examples 4-6 respectively were set up in which a 0, 1, and 2 fold molar excess of pyridoxal phosphate was added. The reactions were allowed to proceed at room temperature for 10 min. and then cooled on ice. Thereafter, a 3-fold molar excess of SC-PEG was added to each and the samples were incubated on ice for 30 min. and at room temperature for 30 min. The reactions were stopped by the addition of excess glycine and dialyzed overnight against 10 mM sodium phosphate pH 6.0. The PEG conjugates were purified by ion exchange chromatography using CM-Spherodex. The 50 mM NaCl eluate was collected for each sample. The PEG #'s for each example, 2.2, 1.6 and 1.7 respectively, were determined by TNBS assay to be similar to that obtained for examples 1-3, indicating that conjugation is unaffected by the presence or absence of added pyridoxal phosphate.

TABLE II

Sample	Pyridoxal Phosphate	IC50 (ug/ml)
Control (unmodified Trichosanthin)	0	0.0001
Example 4	0	0.23
Example 5	1x	0.16
Example 6	2x	0.09

As can be seen in the table, the use of pyridoxal phosphate during conjugation results in much higher activity being retained. For example, the in vitro protein synthesis inhibition activity of example 6, where a 2 fold pyridoxal phosphate excess was included, was more than twice that of example 4 which did not include pyridoxal phosphate.

Examples 7-9

In these examples, three reactions of trichosanthin with 0, 5 and 10-fold molar excess of pyridoxal phosphate were set up in 0.1 M sodium borate pH 9.0. The reactions were allowed to proceed on ice for 10 min. then a three fold molar excess of SC-PEG was added to each reaction mixture. The samples were incubated on ice 30 min. then at room temperature 30 min. The samples were buffer exchanged by repeated concentration and dilution with 10 mM sodium phosphate pH 6.0 and the conjugates were purified by ion exchange chromatography as above. Protein synthesis inhibition assays reported in Table III revealed that PEG-trichosanthin prepared in the presence of a 10-fold excess pyridoxal phosphate, example 9, was 5 times more active than example 7, the conjugate prepared without pyridoxal phosphate. These results were also reflected in an assay for inhibition of HIV replication in an in vitro acute infection system such as that set out in AIDS Research and Human Retroviruses, Volume 6, Number 8 (1990).

TABLE III

Sample	Pyridoxal Phosphate	IC50 (ug/ml)
Control (unmodified Trichosanthin)	0	0.00031
Example 7	0	0.02
Example 8	5x	0.008
Example 9	10x	0.004

TABLE IV

INHIBITION OF HIV REPLICATION

Sample	Pyridoxal Phosphate	IC50 (ug/ml)	Delta
Control (unmodified Trichosanthin)	-	0.0018	
Example 7	0	0.12	67x
Example 8	5x	0.036	20x
Example 9	10x	0.015	8x

While there have been described what are presently believed to be the preferred embodiments of the invention, those skilled in the art will realize that changes in modification may be made thereto without departing from the spirit of the invention. It is intended to claim all such changes and modifications as all within the true scope of the invention.

WE CLAIM:

1. A conjugate comprising a substance having a ribosome inactivating effect and a substantially non-antigenic polymer .

2. The conjugate of claim 1, wherein said substance is bound to said polymer with a carbamate (urethane) linkage.

3. The conjugate of claim 1, wherein said first substance comprises trichosanthin.

4. The conjugate of claim 1, wherein said polymer comprises a poly(alkylene oxide).

5. The conjugate of claim 4, wherein said polymer comprises an alpha-substituted polyalkylene oxide derivative.

6. The conjugate of claim 5, wherein said polymer is selected from the group consisting of polyethylene glycol homopolymers, polypropylene glycol homopolymers, alkyl-capped polyethylene oxides, bis-polyethylene oxides and copolymers or block copolymers of poly(alkylene oxides).

7. The conjugate of claim 6, wherein said polymer comprises poly(ethylene glycol).

8. The conjugate of claim 6, wherein said polymer has a molecular weight of about 200 to about 10,000.

9. The conjugate of claim 8, wherein said polymer has a molecular weight of about 2,000 to about 7,500.

10. The conjugate of claim 8, wherein said polymer has a molecular weight of about 5,000.

11. The conjugate of claim 1, wherein an average of 3-5 strands of said polymer are bound to said substance.

12. A method of preparing a composition having a ribosome inactivating effect, comprising reacting a substance having a ribosome inactivating effect with a substantially non-antigenic polymer under conditions

sufficient to effect conjugation while maintaining at least a portion of the ribosome inactivating effect activity of said substance.

13. The method of claim 12, wherein said polymer is a poly(alkylene oxide).

14. The method of claim 13, wherein said polyalkylene oxide is an alpha-substituted polyalkylene oxide derivative.

15. The method of claim 13, wherein said poly(alkylene oxide) is a polyethylene glycol.

16. The method of claim 11, wherein said reacting step comprises contacting said substance with a molar excess of said polymer relative to said substance.

17. The method of claim 16, wherein said molar excess is from about 2 to about 50 fold.

18. The method of claim 16, wherein said molar excess is from about 3 to about 7 fold.

19. The method of claim 12 further comprising the step of protecting an active lysine site on said substance prior to reacting said substance with said polymer.

20. The method of claim 19, wherein said protecting step comprises contacting said substance with pyridoxal phosphate.

21. The method of claim 20, wherein said pyridoxal phosphate is contacted with said substance in a molar excess.

22. The method of claim 21, wherein said pyridoxal phosphate molar excess is from about 1 to about 15 fold.

23. The method of claim 12, wherein said reacting step is conducted at temperatures of up to 27°C.

24. The method of claim 12, wherein said substance comprises trichosanthin.

25. A method of inhibiting a virus in mammals,

comprising administering an effective amount of the conjugate of claim 1.

26. The method of claim 25, wherein said virus is a human immunodeficiency virus.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/01618

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/62, 37/26, 37/10
US CL : 424/94.3, 88; 514/2, 8; 530/370; 435/1808, 181
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.3, 88; 514/2, 8; 530/370; 435/1808, 181

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Biosis, CA, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCHEMISTRY INTERNATIONAL, Volume 25, No. 6, issued December 1991, H.W. Yeung et al, "Properties of Bromodextran-Trichosanthin: A Comparison with Trichosanthin, an Anti-AIDS Protein," pages 1051-1059, see entire document.	1-26

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 MAY 1994	Date of mailing of the international search report JUN 22 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer LEON LANKFORD Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*